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# Uptake and Release of Bilirubin by Skin

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1. Skin epithelium of albino rat, mouse and guinea pig was shown to accumulate bilirubin from a medium containing free or bound bilirubin. 2. The $K_m$ values for bound bilirubin were $2.22\times10^{-3}$, $1.33\times10^{-3}$ and $9.5\times10^{-4}$ M for rat, mouse and guinea pig respectively and the corresponding $K_m$ values for free bilirubin were $5.2\times10^{-4}$, $4.0\times10^{-4}$, $1.8\times10^{-4}$ M; the $V_{max.}$ values of bound and free bilirubin were unchanged. 3. The uptake showed saturation kinetics. Bound bilirubin was released together with serum proteins. Free bilirubin bound to skin was not released into the medium. 4. Freezing and thawing of skin epithelium did not cause any significant lowering of the uptake of bilirubin but heat-denatured skin epidermis took up only 50% of the bound bilirubin or free bilirubin taken up by control unheated skin epithelium. 5. The uptake of free and bound bilirubin was prevented by $HgCl_2$ but not by sodium arsenate, NaCN, NaF, cycloheximide, 2,4-dinitrophenol or iodoacetate. 6. Most of the free bilirubin was bound to the lipids or lipoprotein fraction of skin epithelium and could be extracted by solvents. 7. Rat skin showed the highest accumulation and efflux of bilirubin.

Yellow discolouration of skin occurs in hyperbilirubinaemia of the newborn and in older people. A glucuronate-transfer mechanism that can convert bilirubin into its glucuronide is present in the epithelial layers of the intestines (Stevensen & Dutton, 1962) and the existence of an uptake mechanism for bilirubin in fat-cells, epidermal cells and nervous tissue has been suggested (Behrman & Hsia, 1969). It is presumable from these reports that the skin is equipped with a mechanism for the uptake, concentration and metabolism of bilirubin. As part of an investigation on the detoxication of bilirubin by phototherapy in hyperbilirubinaemia of the newborn (Bajpai *et al.*, 1973), the present study deals with the kinetics of uptake and efflux of bilirubin by skin segments of the albino rat, mouse and guinea pig.

## Experimental

### *Chemicals and reagents*

Crystalline bilirubin for biochemistry was a product of E. Merck, Darmstadt, Germany, and gave $\epsilon$ 58000–61000 litre·mol$^{-1}$·cm$^{-1}$ at 450nm in chloroform. Sephadex G-10 (particle size 40–60,) was obtained from Pharmacia, Uppsala, Sweden. Cyanogum $R_{41}$ (mixture of acrylamide and *NN'*-methylenebisacrylamide) was from American Cyanamide Co., Wayne, N.J., U.S.A., *NNN'N'*-tetramethylethylenediamine from Eastman Organic Chemicals, Rochester, N.Y., U.S.A., Dextran Blue from Serva Fine Biochemica, Heidelberg, Germany, Tris (ultrapure) from Mann Research Laboratories Inc., New York, N.Y., U.S.A. and bovine serum albumin, human serum albumin and iodoacetate from Sigma Chemical Co., St. Louis, Mo., U.S.A. *p*-Iodoaniline was prepared in the laboratory by standard methods (Vogel, 1968). The other chemicals used were of AnalaR grade.

### *Animals*

The animals used were drawn from the Stock Colony of the Central Drug Research Institute, Lucknow.

### *Methods*

*Preparation of skin segment.* Animals were starved overnight and portions on the abdominal region shaved with an electric shaver. The animals were killed by decapitation, skin pieces were cut out (approx. 5cm$^2$) and transferred to chilled 150mM-KCl. The procedure of Sahib & Krishna Murti (1969) based on that of Van Scott (1952) was used for separating epidermal layers.

*Bilirubin solution.* Bilirubin solution was prepared by quickly dissolving 20mg of the crystalline compound in 0.5ml of 0.1 M-NaOH and making the volume up to 10ml with distilled water. The pH was immediately adjusted to about 9. The solution was

kept in the dark at about 5°C, its $\epsilon$ at 440nm was 51200 litre·mol$^{-1}$·cm$^{-1}$ and it was used within 1h of preparation.

*Human serum saturated with bilirubin.* Blood taken from donors of the Blood Bank of K.G. Medical College, Lucknow, was allowed to clot and the serum recovered. Portions (10–20ml) of serum were mixed with 30–60mg of bilirubin dissolved in a few drops of 0.1M-NaOH and the pH of the suspension adjusted to 7.5 with 3vol. of Krebs–Ringer phosphate buffer of the following composition: NaCl, 122mM; KCl, 5.0mM; $MgSO_4$, 1.2mM; $CaCl_2$, 1.0mM; $Na_2HPO_4$, 16mM. The solution was placed in 12ml plastic centrifuge tubes and centrifuged in a no. 40 rotor of a Spinco model L preparative ultracentrifuge at 39000rev./min (100328*g*; $r_{av.}$ 5.9cm) for 1h at 5°C. The supernatant was saved and used as serum saturated with bilirubin.

Alternatively the serum–buffer mixture was filtered through a column (10cm×1.2cm) of Sephadex G-10 previously equilibrated with Krebs–Ringer phosphate buffer, pH7.5. The filtrate was used as serum saturated with bilirubin.

*Uptake and efflux studies.* To flasks (25ml Erlenmyer) each containing 0.5g portions of epidermal segments and 5ml of Krebs–Ringer buffer, pH7.5, was added serum saturated with bilirubin or an aqueous solution of bilirubin to a final concentration of 0.427mM. With their mouths closed with aluminium foil, the flasks were shaken in the dark in a metabolic shaker (60 strokes/min, amplitude 2cm) for the required period. The skin segments were then washed free from medium, homogenized and used for the determination of bilirubin. In the efflux studies, skin segments preincubated in serum saturated with bilirubin or aqueous solution of bilirubin were washed twice with chilled Krebs–Ringer buffer, pH7.5, and then transferred to fresh lots of Krebs–Ringer buffer and shaken again for 2h. The medium was recovered and used for the assay of effluxed bilirubin and proteins.

*Preparation of homogenates.* The epidermal strips were suspended in 150mM-KCl and kept frozen at −18°C and allowed to thaw at 40–45°C. Freezing and thawing were repeated at least four times; the suspension was then made up to 9vol. with 150mM-KCl and homogenized in a power-driven Potter–Elvehjem glass homogenizer fitted with a Teflon pestle or in a Pyrex (A. H. Thomas, Philadelphia, Pa., U.S.A.) no. 7725 glass homogenizer. By running the homogenizer at maximum speed for 20min one could get a fairly uniform homogenate by the above procedure. Alter-

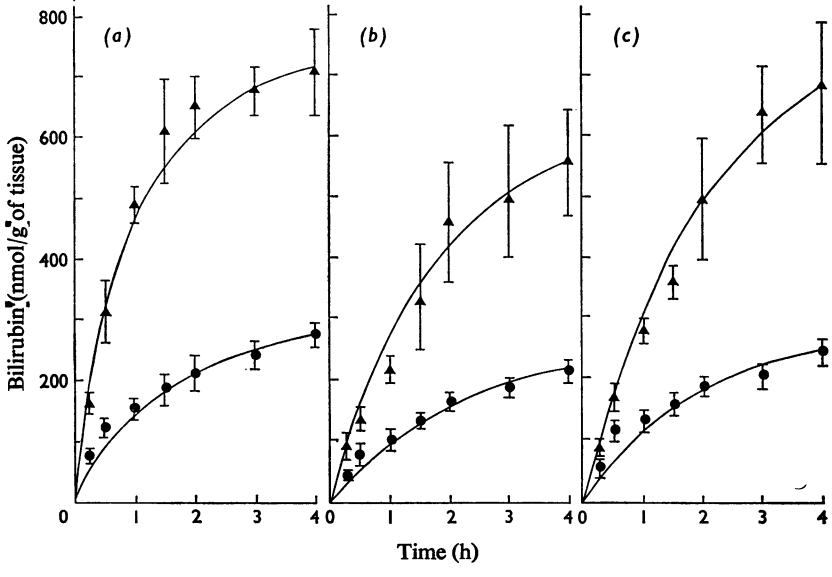


Fig. 1. *Uptake of bilirubin by rat, mouse and guinea-pig skin epidermis with time*

▲, Free bilirubin; ●, bound bilirubin. Skin epithelium strips (500mg) were incubated at 37°C in 5ml of incubation medium containing 0.427mM-bound bilirubin or 0.427mM-free bilirubin. At various time-intervals uptake of bilirubin was determined as described in the Experimental section. The vertical bars are the standard deviation (S.D.) of the mean values from at least three experiments. (*a*) Rat; (*b*) guinea pig; (*c*) mouse.

natively the epidermal segments were thrown into liquid air, the frozen pellets thawed at room temperature and the operation repeated to get a pulpy mass that could be homogenized in the Potter–Elvehjem homogenizer as above.

*Heat-treatment.* Epidermal segments in Krebs–Ringer buffer, pH7.5, were kept at the requisite temperature for 5min.

*Determination of bilirubin.* Free bilirubin in crude homogenates or efflux media was determined by the procedure described by Van Roy *et al.* (1971) which gave over 94% recovery of bilirubin added to skin homogenates. The azo pigments were extracted with butyl acetate. The concentration of bilirubin in medium was determined by the micro method of Malloy & Evelyn (1937).

*Protein.* Protein in efflux medium or serum was measured by the method of Lowry *et al.* (1951) with dry bovine serum albumin as standard.

*Identification of effluxed proteins.* The proteins released by 5g of rat epidermis were pooled, dialysed overnight against glass-distilled water and freeze-dried. Polyacrylamide-gel electrophoresis of the proteins was done as described by Clarke (1964). Globulin present in the proteins was identified by immunodiffusion against Coomb's serum as described by Kabat & Mayer (1961).

*Preparation of lipid micelles.* Rat skin epithelial

Table 1. *Effect of tissue concentration on uptake of bilirubin by rat, mouse and guinea pig*

Epidermal tissue (200–1000mg) was incubated in 5.0ml of incubation medium (pH7.5) containing 0.427mM-bound bilirubin or 0.427mM-free bilirubin at 37°C for 2h. The uptake of bilirubin was determined as described in the Experimental section.

| Wet wt. of tissue (g) | Bound bilirubin (nmol) | | | Free bilirubin (nmol) | | |
|---|---|---|---|---|---|---|
| | Rat | Guinea pig | Mouse | Rat | Guinea pig | Mouse |
| 0.20 | 36 | 34 | 34 | 128 | 80 | 108 |
| 0.40 | 78 | 69 | 72 | 255 | 168 | 204 |
| 0.60 | 126 | 100 | 106 | 444 | 274 | 336 |
| 0.80 | 168 | 136 | 144 | 544 | 304 | 432 |
| 1.00 | 210 | 176 | 170 | 670 | 400 | 540 |

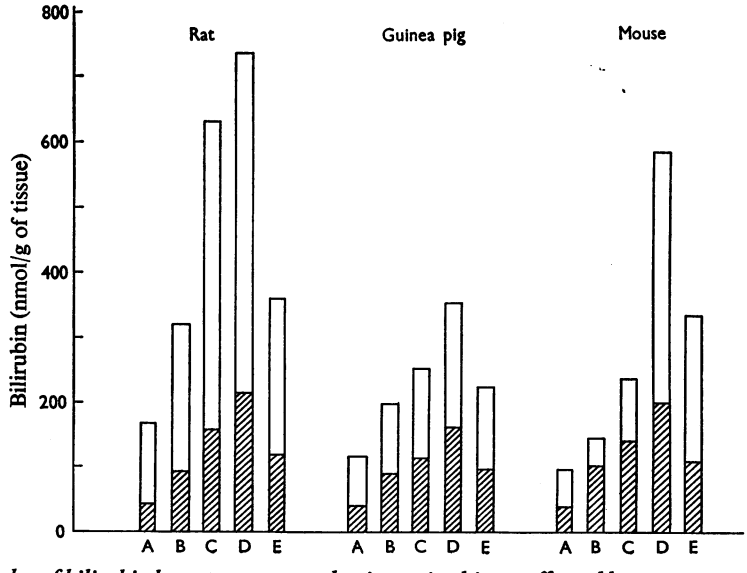


Fig. 2. *Uptake of bilirubin by rat, mouse, and guinea-pig skin as affected by temperature of incubation*

Epidermal strips (500mg) were incubated at A (4±1°C), B (15±1°C), C (25±1°C), D (37±1°C), E (52±1°C) in 5ml of incubation medium containing 0.427mM-bound bilirubin or 0.427mM-free bilirubin for 2h. Each value plotted represents the mean of two or more separate experiments. □, Free bilirubin; ■, bound bilirubin.

homogenate was extracted with chloroform – methanol (2:1, v/v) mixture by the procedure of Folch *et al.* (1957). Samples of total lipids (10mg) dispersed in 50mM-sodium phosphate buffer, pH7.5, 50mM-Tris–HCl buffer, pH7.5, were exposed to ultrasonic waves for 10min at 20kHz (Mullard, London W.C.1, U.K.). After sonic disruption samples were centrifuged at 5°C at 100328*g* ($r_{av.}$ 5.9) for 60min. The supernatant was used as the source of lipid micelles (Kapoor *et al.*, 1972).

## Results

### *Bilirubin binding capacity of human serum*

At pH7.5 252mg of bilirubin were bound/litre of serum. Analysis of 12 different sera gave bilirubin binding capacity of 220–274mg of bilirubin/100ml ($26.0\times10^{20}$ molecules/litre of serum). The binding capacity increased when the pH was adjusted from 6 to 8.5. Therefore the serum used for providing bound bilirubin contained the above concentration of bound bilirubin.

### *Uptake of bilirubin by epidermal segments*

The uptake of bilirubin as free bilirubin and as bound bilirubin by rat, mouse and guinea-pig epidermal segments as affected by time of incubation is illustrated in Fig. 1. The effect of concentration of tissue on the uptake is illustrated in Table 1, the effect of temperature of incubation in Fig. 2 and the effect of pH on the uptake in Fig. 3.

The rate of uptake as affected by concentration of bilirubin in the medium is shown in Fig. 4. The $K_m$ values for bound bilirubin were $2.22\times10^{-3}$, $1.33\times10^{-3}$ and $9.5\times10^{-4}$M for rat, mouse and guinea-pig skin respectively and the $K_m$ values for free bilirubin were $5.2\times10^{-4}$, $4.0\times10^{-4}$ and $1.8\times10^{-4}$M respectively.

### *Effect of inhibitors*

Sodium arsenate, NaCN, NaF, dinitrophenol and cycloheximide did not inhibit the uptake of bilirubin (Table 2). $HgCl_2$ (10mM), however, caused 50% inhibition of the uptake.

### *Efflux of bilirubin from skin*

Results of efflux experiments are represented in Fig. 5. Mean rates of efflux were 0.81, 0.80 and 0.70nmol/min per g for rat, mouse and guinea-pig skin respectively. The efflux was fast initially, up to 30min.

The efflux medium contained, together with bilirubin, proteins, which were identified by polyacrylamide-gel electrophoresis or by immunogel-diffusion technique as serum albumin and globulin (results not given).

### *Effect of preheating skin on bilirubin uptake*

The effect of preheating skin segments to different temperatures on its capacity to concentrate bilirubin from the medium is illustrated in Table 3 and Fig. 6.

### *Sites of binding of bilirubin on skin*

Since the efflux of bilirubin from skin was mostly accountable as bilirubin bound to serum proteins, it was of interest to determine the amount bound and held in the extravascular space by skin components other than serum proteins. For this, skin segments incubated in the presence of free and bound bilirubin were subjected to freezing and thawing and heat-treatment and solvent extraction and the results are summarized in Tables 4 and 5.

### *Binding of bilirubin to isolated skin lipid micelles*

Skin lipid micelles prepared from rat skin epithelium were allowed to interact with aqueous bili-

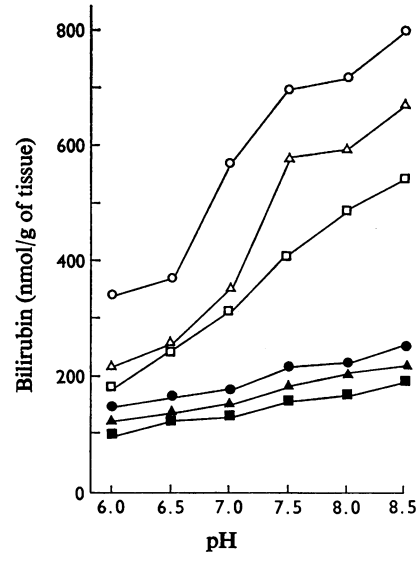


Fig. 3. *Uptake of bilirubin as affected by the pH of the medium*

Epidermal strips (500mg) were incubated at 37°C for 2h in 5ml of incubation medium containing 0.427mM-bound or -free bilirubin. Uptake and determination of bilirubin were followed as described in the Experimental section. Each value plotted represents the mean of two or more separate experiments. ○, Free bilirubin in rat skin; △, free bilirubin in mouse skin; □, free bilirubin in guinea-pig skin; ●, bound bilirubin in rat skin; ▲, bound bilirubin in mouse skin; ■, bound bilirubin in guinea-pig skin.

rubin and the $E_{440}$ changes were followed. Results are presented in Fig. 7.

## Discussion

Free bilirubin is relatively insoluble in aqueous medium (Burnstine & Schmid, 1962) and like other weakly acidic substances is transported by serum bound to the proteins (Klatskin & Bungards, 1950; Ostrow & Schmid, 1963; Cooke & Robert, 1969). The relative distribution of bilirubin in various tissues would depend therefore on the bilirubin binding capacity of serum constituents.

There is increasing evidence that liver is preferentially able to transfer bilirubin from plasma to parenchymal cells (Brown *et al.*, 1965; Bernstein *et al.*, 1966) and certain anion-binding proteins located in the cytoplasm are involved in the uptake (Levi *et al.*, 1969; Fleischner & Arias, 1970). Hargreaves & Price (1966) studied the uptake of bilirubin by liver and kidney slices *in vitro*.

The present studies provide unequivocal evidence

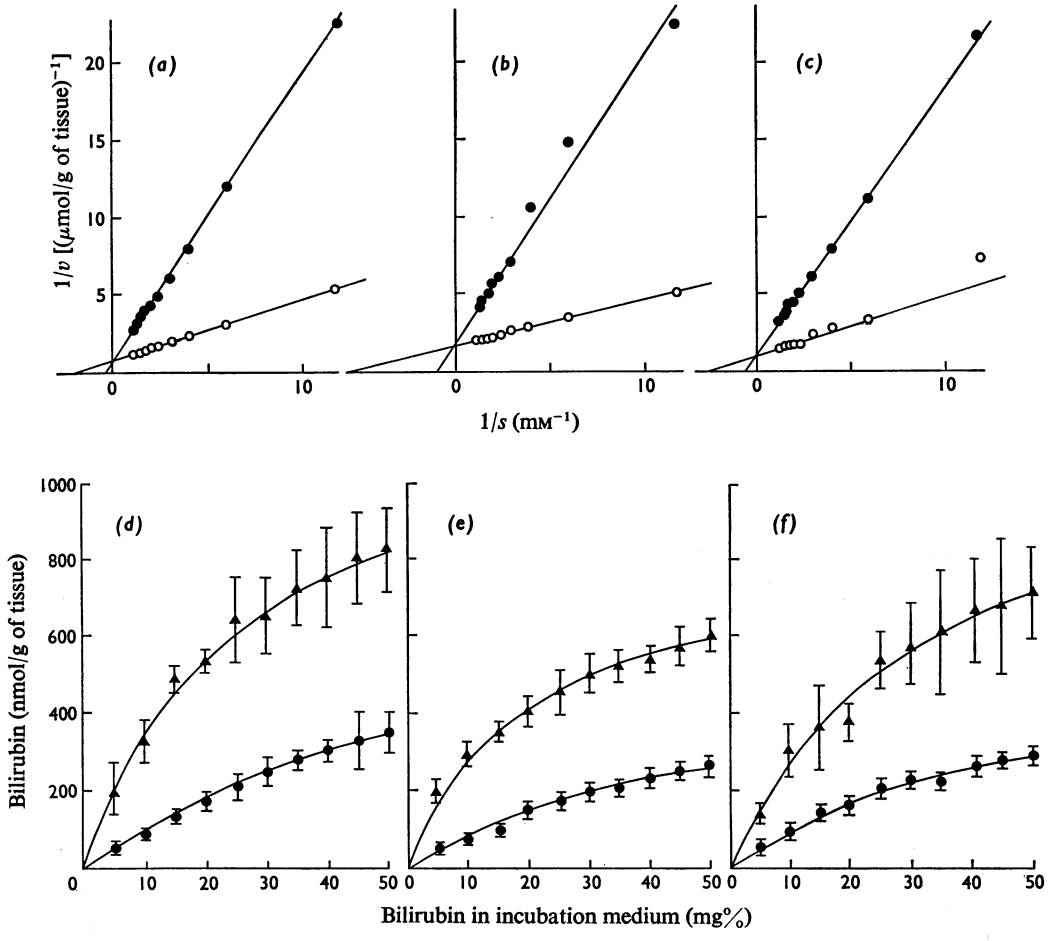


Fig. 4. *Uptake of bilirubin from (d) rat, (e) guinea-pig and (f) mouse skin epithelium and Lineweaver–Burk double-reciprocal plots of the uptake of bilirubin by (a) rat, (b) guinea-pig and (c) mouse skin epithelium showing the graphical calculations of $K_m$ values*

(*a*)–(*c*) ●, Uptake from bound-bilirubin medium; ○, from free-bilirubin medium. (*d*)–(*f*) ▲, Free bilirubin; ●, bound bilirubin. Skin strips (500mg) were incubated in increasing 5–50mg % (0.0854–0.854mM) concentrations of bound and free bilirubin in incubation medium (pH7.5) at 37°C for 2h. The uptake and determination of bilirubin were followed as described in the Experimental section. The bars represent the S.D. of the mean values from at least three experiments.

Table 2. *Effect of inhibitors on the uptake of bilirubin by rat skin epithelium*

Skin epithelium strips (500mg) were incubated at 37°C for 2h in 5ml of incubation medium (Krebs–Ringer buffer, pH7.5) containing 0.427mM-bound or -free bilirubin as described in the Experimental section. Inhibitors were added to the indicated final concentrations. Each result is the mean of the values from two separate experiments.

| Inhibitor | Concentration (mM) | Bilirubin (nmol/g of tissue) Bound bilirubin | Free bilirubin |
|---|---|---|---|
| Nil | | 204 | 780 |
| Sodium arsenate | 0.1 | 186 | 810 |
| | 1.0 | 214 | 790 |
| | 10.0 | 220 | 740 |
| NaCN | 0.1 | 200 | 810 |
| | 1.0 | 186 | 770 |
| | 10.0 | 203 | 740 |
| NaF | 0.1 | 200 | 760 |
| | 1.0 | 194 | 720 |
| | 10.0 | 180 | 740 |
| $HgCl_2$ | 0.1 | 180 | 800 |
| | 1.0 | 160 | 624 |
| | 10.0 | 100 | 410 |
| Cycloheximide | 0.1 | 206 | 780 |
| | 1.0 | 204 | 830 |
| | 10.0 | 200 | 840 |
| 2,4-Dinitrophenol | 0.1 | 190 | 766 |
| | 1.0 | 192 | 700 |
| | 10.0 | 196 | 880 |
| Iodoacetic acid | 0.1 | 206 | 820 |
| | 1.0 | 184 | 830 |
| | 10.0 | 160 | 744 |

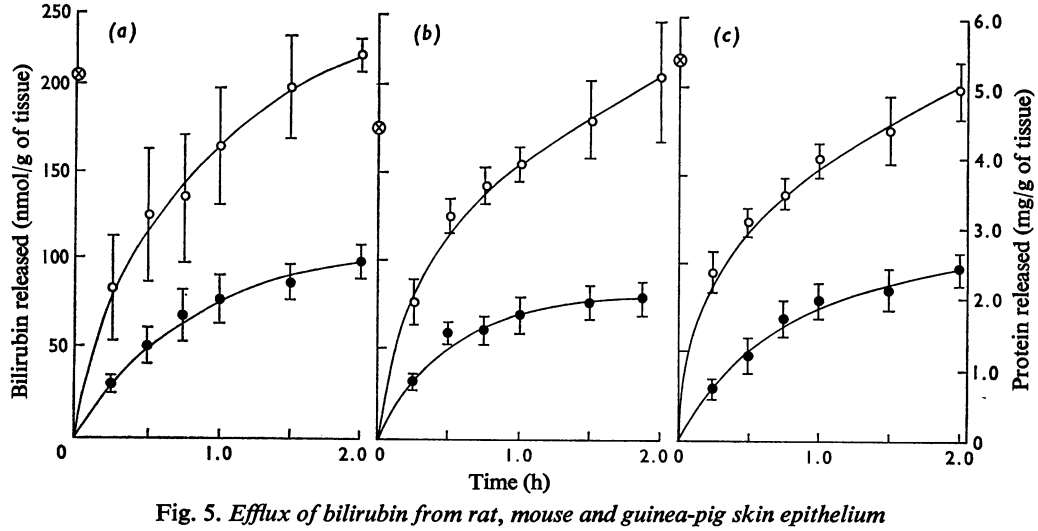


Fig. 5. *Efflux of bilirubin from rat, mouse and guinea-pig skin epithelium*

Tissue (500mg) was incubated in 5ml of incubation medium containing 0.427mM-bound bilirubin (containing 36.5mg of serum protein) at 37°C for 2h, washed free from medium and then incubated in 5ml of Krebs–Ringer buffer (pH7.5). The release of bilirubin (●) and protein (○) were followed for 2h. The bars represent the S.D. of the mean values from three or more separate experiments. (*a*) Rat; (*b*) guinea pig, (*c*) mouse. ⊗, Initial bilirubin content of skin strips.

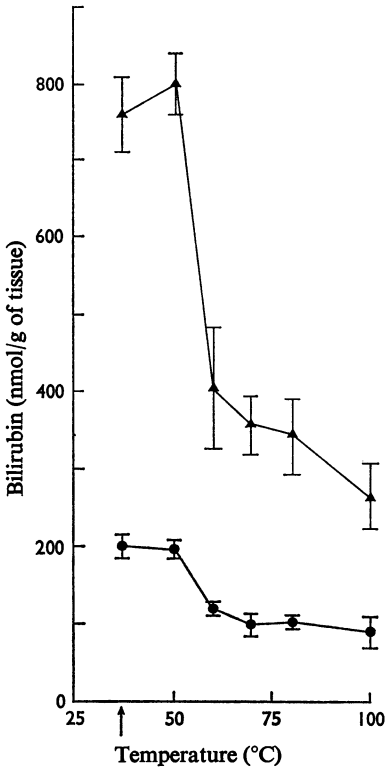


Fig. 6. *Effect of preheating on the uptake of bilirubin by rat skin*

▲, Free bilirubin; ●, bound bilirubin. Tissue (500mg) was denatured by heating at 37°, 50°, 60°, 80° and 100°C for 5min, cooled to room temperature (28°C) and used in the uptake studies at 37°C in 5ml of incubation medium (pH7.5) containing 0.427mM-bound or 0.427mM-free bilirubin for 2h. The bars represent the S.D. of the mean values from three or more separate experiments. The arrow shows the incubation temperature.

to show that epidermal segments of the three mammals studied can pick up bilirubin from the medium when the latter is present either in the free form or in the bound form. The bound form is presumably exchanged with serum proteins present in skin as is evident from the efflux studies. The major part of the uptake is, however, as free bilirubin.

Denaturation experiments indicate that there are sites located on the skin itself that account for the large uptake of free bilirubin. Part of this uptake could be by lipids present in skin, as is evident from binding of bilirubin to skin lipids *in vitro* and the fact that heating of skin strips after uptake experiments releases some oily substances containing bilirubin. Extraction with solvent also removes most of the bilirubin picked up by skin segments. This is not surprising from the observation that erythrocyte membrane lipids can bind bilirubin (Kapoor *et al.*, 1972).

The major part of free bilirubin picked up by epidermis goes into some tight binding with, presumably, the lipoproteins of skin. Stenhagen & Rideal (1939) showed that binding can take place between phenolic hydroxyl groups of bilirubin and the primary amino groups of proteins of serum. In the case of serum albumin, the free amino groups are presumably contributed by its 55 lysine residues and when these are blocked by methylisourea, binding is very much diminished (Martin, 1949). However, immunoglobulin G, which has a high lysine content, does not bind bilirubin and hence properties other than free lysine residues of the protein molecule must be involved in the binding.

The binding of bilirubin to skin constituents takes place within a pH range of 6–9. This behaviour is somewhat different from that of albumin, the binding property of which is limited to a narrow pH range (Hargreaves, 1968). The binding of free bilirubin to sites on epidermis is sensitive to temperature and on heating from 50° to 75°C more than two-thirds of the original activity is lost. In contrast, heat-denatured

Table 3. *Effect of heating of skin epithelium strips on the uptake of bilirubin*

The epidermal segments were kept at 100°C for 5min, cooled and used in uptake studies at 37°C. Medium contained 0.427mM-bilirubin bound to serum or 0.427mM-bilirubin in aqueous medium. Uptake was allowed to proceed in an incubation medium as described in the Experimental section. Each value represents the mean of two to four separate experiments.

| | Bilirubin (nmol/g of tissue) | | | | | |
|---|---|---|---|---|---|---|
| Experimental animal ... | Rat | | Mouse | | Guinea pig | |
| | Bound | Free | Bound | Free | Bound | Free |
| Normal skin epithelium strips | 208 | 690 | 190 | 594 | 150 | 410 |
| Heated skin epithelium strips | 85 | 300 | 88 | 244 | 72 | 150 |

Table 4. *Effect of freezing and thawing of rat skin epithelium on the uptake of bilirubin*

Epidermal strips were frozen at −180°C (liquid air) and thawed at room temperature (28°C); freezing and thawing were repeated four times. The uptake and measurements of bilirubin were followed as described in the Experimental section. The results show the amount of bilirubin held by the tissue after each treatment. Freezing and thawing do not cause any significant lowering in the uptake of bilirubin, but heating of such frozen and thawed tissue decreased uptake of both bound and free bilirubin by above 50%. Each value represents the mean of two to four separate experiments.

| Treatments | Bilirubin (nmol/g of tissue) | |
|---|---|---|
| | Bound | Free |
| Control | 191 | 743 |
| Frozen and thawed | 155 | 703 |
| Frozen, thawed and centrifuged | 174 | 648 |
| Frozen, thawed, centrifuged and heated | 95 | 370 |

Table 5. *Effect of freezing and thawing on rat skin epithelium allowed to accumulate bilirubin*

Epidermal strips were allowed to accumulate bilirubin as described in the Experimental section and then frozen and thawed. The amount of bound bilirubin present in skin before freezing and thawing was 200nmol/g and free bilirubin 670nmol/g. Each value is the mean of two or more separate experiments.

| Bilirubin accumulated by skin epithelium with | Bilirubin (nmol/g of tissue or released in medium by g of tissue) |
|---|---|
| Bound bilirubin | 92 |
| Free bilirubin | 10 |

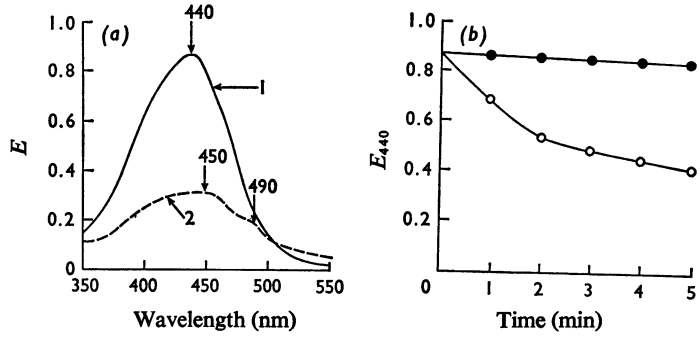


Fig. 7. *Absorbance change of bilirubin spectrum on binding to skin epithelium lipid micelles*

(*a*) Curve 1. Absorption spectra of 17.1 $\mu$M-bilirubin in 50mM-sodium phosphate buffer, pH7.5, 50mM-Tris–HCl buffer, pH7.5. Curve 2. Same as curve 1+20 $\mu$g of lipid micelles/ml. (*b*) Decrease of $E_{440}$ of bilirubin in the presence of skin lipid micelles as affected by time. ●, No lipid micelles; ○, +20 $\mu$g of lipid micelles/ml.

serum protein is reported to have a high affinity for bilirubin (Nosslin, 1960); but the behaviour of heat-denatured proteins of skin in this respect appears to be quite different.

Hargreaves & Price (1966) have reported that NaF and NaCN do not inhibit the uptake of bilirubin by liver slices suggesting that oxidative enzymes may not be involved in the uptake process. Heat-denatured liver slices have been shown to take up more conjugated bilirubin than normal slices. Temperatures above 55–60°C caused greater denaturation of liver slices and greater uptake of bilirubin perhaps owing

to exposure of more bilirubin-binding sites (Hargreaves & Price, 1966; Mackay & Martin, 1957). The behaviour of skin was in contrast with this, because above 50°C there was a sharp decline in the uptake of both free and bound bilirubin.

Bilirubin is closely bound to plasma albumin and as such is transported to the liver or other tissues from the site of its formation. The intracellular localization of bilirubin would depend on the presence of intracellular binding forces of sufficient strength to compete with the binding of albumin present in the extracellular compartment. That such forces do exist in skin is apparent from the present study.

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